

**Enzymatic Assay of AGARASE  
(EC 3.2.1.81)**

**PRINCIPLE:**

Agar + H<sub>2</sub>O  $\xrightarrow{\text{Agarase}}$  Reducing Sugar (measured as galactose)

**CONDITIONS:** T = 43°C, pH = 6.0, A<sub>540nm</sub>, Light path = 1 cm

**METHOD:** Colorimetric

**REAGENTS:**

- A. 50 mM Potassium Phosphate Buffer, pH 6.0 at 43°C  
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.0 at 43°C with 1 M KOH.)
- B. 1.5% (w/v) Agar Substrate Solution (Agar)  
(Prepare 50 ml in Reagent A using Agar, Sigma Prod. No. A-7002.)<sup>1</sup>
- C. Agarase Enzyme Solution  
(Immediately before use, prepare a solution containing 20 - 40 units/ml of Agarase in cold Reagent A.)<sup>2</sup>
- D. 16 mM Copper Sulfate, 1.3 M Sodium Sulfate, 226 mM Sodium Carbonate, 190 mM Sodium Bicarbonate, and 43 mM Sodium Potassium Tartrate Solution (Copper Soln)  
(Prepare 1 liter in deionized water using Cupric Sulfate Pentahydrate, Sigma Prod. No. C-7631, Sodium Sulfate, Anhydrous, Sigma Prod. No. S-9627, Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127, Sodium Bicarbonate, Sigma Prod. No. S-8875, and Sodium Potassium Tartrate Tetrahydrate, Sigma Prod. No. S-2377.)<sup>3</sup>
- E. 40 mM Molybdic Acid, 19 mM Arsenic Acid and 756 mM Sulfuric Acid Solution (Ars-Mol)  
(Prepare 1 liter in deionized water using Molybdic Acid, Ammonium Salt, Tetrahydrate, Sigma Prod. No. M-0878, Arsenic Acid, Sodium Salt, Sigma Prod. No. A-6756, and Sulfuric Acid, Sigma Prod. No. S-1526.)<sup>4</sup>

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**REAGENTS:** (continued)

- F. 0.5 mg/ml Galactose Standard Solution (Gal Std)  
(Prepare 10 ml in Reagent A using D(+)Galactose, Sigma Prod. No. G-0750.)

**PROCEDURE:**

Pipette (in milliliters) the following reagents into suitable 4 dram vials:

	<u>Test</u>	<u>Blank</u>
Reagent B (Agar)	4.00	4.00

Equilibrate to 43°C. Then add:

Reagent C (Enzyme Solution)	2.00	-----
Reagent A (Buffer)	-----	2.00

Mix by swirling and incubate at 43°C for 30 minutes. At times 15, 20, 25, and 30 minutes, remove a 1.00 ml aliquot.<sup>5</sup>

**COLORIMETRIC DETERMINATION:**

Pipette (in milliliters) the following into suitable tubes (equilibrated to 43EC):

	<u>T<sub>15</sub></u> <u>Test</u>	<u>T<sub>20</sub></u> <u>Test</u>	<u>T<sub>25</sub></u> <u>Test</u>	<u>T<sub>30</sub></u> <u>Test</u>	<u>Test</u> <u>Blank</u>
T <sub>15</sub> Test Solution	1.00	-----	-----	-----	-----
T <sub>20</sub> Test Solution	-----	1.00	-----	-----	-----
T <sub>25</sub> Test Solution	-----	-----	1.00	-----	-----
T <sub>30</sub> Test Solution	-----	-----	-----	1.00	-----
Test Blank	-----	-----	-----	-----	1.00
Reagent D (Copper Soln)	1.00	1.00	1.00	1.00	1.00

Immediately mix by swirling. Place a marble over the top of the tubes and transfer the tubes to a boiling water bath. Incubate for 10 minutes. Remove from the boiling water bath and allow to cool to room temperature.<sup>6</sup>  
Then add:

Reagent E (Ars-Mol)	1.00	1.00	1.00	1.00	1.00
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**COLORIMETRIC DETERMINATION:** (continued)

Shake or vortex until the foaming stops and any orange colored precipitate present is dissolved. Then add (in milliliters):

	<u>T<sub>15</sub></u> <u>Test</u>	<u>T<sub>20</sub></u> <u>Test</u>	<u>T<sub>25</sub></u> <u>Test</u>	<u>T<sub>30</sub></u> <u>Test</u>	<u>Test</u> <u>Blank</u>
Deionized Water	10.00	10.00	10.00	10.00	10.00

Mix by swirling<sup>7</sup>, and transfer to suitable cuvettes. Obtain the A<sub>540nm</sub> for both Test and Blank, using a suitable spectrophotometer.

Standards:

Pipette (in milliliters) the following reagents into suitable tubes (Mix after each addition):

	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std 4</u>	<u>Std 5</u>	<u>Std</u> <u>Blank</u>
Reagent A (Buffer	0.95	0.80	0.60	0.40	---	1.00
Reagent F (Gal std)	0.05	0.20	0.40	0.60	1.00	---
Reagent D (Copper Soln)	1.00	1.00	1.00	1.00	1.00	1.00

Immediately mix by swirling. Place a marble over the top of the tubes and transfer the tubes to a boiling water bath. Incubate for 10 minutes. Remove from the boiling water bath and allow to cool to room temperature. Then add:

	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std</u> <u>Blank</u>
Reagent E (Ars-Mol)	1.00	1.00	1.00	1.00

Shake or vortex until the foaming stops and any orange colored precipitate present is dissolved. Then add:

Deionized Water	10.00	10.00	10.00	10.00
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Mix by swirling<sup>7</sup>, and transfer to suitable cuvettes. Obtain the A<sub>540nm</sub> for the Standards and Standard Blank, using a suitable spectrophotometer.

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**CALCULATIONS:**

$$A_{540\text{nm}} \text{ Standard} = A_{540\text{nm}} \text{ Standard} - A_{540\text{nm}} \text{ Standard Blank}$$

Prepare a standard curve by plotting the  $A_{540\text{nm}}$  of the Standard versus micrograms of D-Galactose.

**Sample Determination:**

$$A_{540\text{nm}} \text{ Sample} = A_{540\text{nm}} \text{ Test} - A_{540\text{nm}} \text{ Blank}$$

Determine the micrograms of D-Galactose liberated using the standard curve.

$$\text{Units enzyme/ml} = \frac{(\text{micrograms of D-Galactose liberated}) (6.0) (\text{df})}{(2.0) (T)}$$

6.0 = total volume (in milliliters) of assay

df = Dilution factor

2.0 = Volume (in milliliters) of enzyme used

T = Time (in minutes) at which Reagent D was added

$$\text{Unit/mg solid} = \frac{\text{unit/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Unit/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

**UNIT DEFINITION:**

One unit will produce 1.0  $\mu\text{g}$  of reducing sugar (measured as D-galactose) from agar per minute at pH 6.0 at 43°C.

**FINAL ASSAY CONCENTRATIONS:**

In a 6.00 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 1.0% (w/v) agar and 40 - 80 units agarase.

**REFERENCE:**

Morrice, L. M. et al. (1983) *European Journal of Biochemistry* **135**, 553-558

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**NOTES:**

1. Heat to boiling with stirring to dissolve. Cool to 43°C for the assay. Reagent will solidify below 43°C.
2. Preheat enzyme solution to 43°C immediately before adding to Reagent B (agar) or it will cause the agar to solidify.
3. Sodium Sulfate, Sodium Carbonate, and Sodium Potassium Tartrate are dissolved in approximately 500 ml of deionized water. Cupric Sulfate is dissolved in approximately 100 ml of deionized water and slowly added to the above solution to avoid precipitation. Sodium Bicarbonate is dissolved first in deionized water and then added to the above solution. Dilute the solution to 1 liter. Store in an amber bottle at room temperature and avoid exposure to direct light. If a precipitate forms the solution may be used after the precipitate is allowed to settle. The solution may also be used without allowing the precipitate to settle, but the reaction mix will need to be filtered prior to reading the absorbance of the spectrophotometer.
4. Molybdic Acid is dissolved in approximately 300 ml of deionized water. Add Sulfuric Acid slowly. Caution, this is an exothermic reaction! Arsenic Acid is dissolved in approximately 300 ml of deionized water and is added to the above solution. The solution is diluted to a total volume of 1 liter and incubated at 37°C for 48 - 72 hours. Store in an amber bottle at room temperature and avoid exposure to direct light. The solution expires six months after preparation. If a precipitate forms the solution may be used after the precipitate is allowed to settle. The solution may also be used without allowing the precipitate to settle, but the reaction mix will need to be filtered prior to reading the absorbance on the spectrophotometer.
5. Removing the pipet tip from the pipettor and leaving the tip in the reaction mix throughout the incubation allows the tip to equilibrate to the appropriate temperature. Using this same tip to remove 1 ml aliquots at all the time points reduces the chance for the agar to solidify in the tip.

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**NOTES:** (continued)

6. Do not place in an ice bath to cool to room temperature, it will cause the agar to solidify.
7. If a precipitate is present it can be removed by filtering through an 0.8  $\mu\text{m}$  syringe filter.
8. This assay is based on the cited reference.
9. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

**This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.**